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RECOMBINATION OF INFLUENZA A VIRUS STRAINS : EFFECT ON PATHOGENICITY

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ABSTRACT

Influenza viruses can recombine genetic information, and progeny virus can be selected for desired genetic traits. A newly isolated strain can develop the ability to grow to higher titer in embryonated eggs by acquiring this trait from the A0PR8(H0N1) strain, and can be selected by the terminal dilution technique and by treatment with anti-H0N1 antisera. In addition to acquiring the ability to grow to high titer, surface antigens may also be transferred, and the progeny can be selected by immunologic methods. It appeared likely that other genes, including those coding for virulence or attenuation might also be exchanged during the recombination procedure. We explored this question by infecting mice with recent strains of H3N2 influenza virus, and with some of their progeny which had been selected for high virus yields from eggs, after recombination with the A0PR8(H0N1) virus, a strain known to be virulent for mice.

Influenza (H3N2) virus strains A/Scotland, A/Port Chalmers and A/England which were isolated and passaged in embryonated eggs did not cause death when administered intranasally to three-week-old Swiss mice; however, after recombination with the A/PR8(H0N1) virus, these viruses become lethal in mice. This acquisition of virulence appears to be secondary to exchange of genetic information from the parent A0PR8 virus. Virus isolated from mice infected with these recombinants is antigenically H3N2, the mice develop anti-H3N2 antibodies.

Influenza A viruses readily recombine genetic information during mixed infections. This phenomenon has been studied extensively by Kilbourne (1960, 1967, 1969) and others (1, 5). Progeny viruses possessing various traits as a result of gene reassortment can be selected. Characteristics such as virulence which are determined by several genes are not likely to be expressed to the same degree in the hybrid progeny as in the parent, and new characteristics have been detected after recombination, so the traits of the recombinants must be characterized in order to ascertain the outcome of this genetic reassortment.

The identification and selection of antigenic hybrids, i.e., recombinants with the hemagglutinin of one parent and the neuraminidase of the other parent can be accomplished by using specific antisera prepared with isolated viral proteins. Selection of high yielding strains of progeny virus can be performed by terminal dilution techniques, where only the highest growing progeny virus will be present.

There are no such facile methods for selecting progeny virus with a desirable degree of attenuation. Thus, after clones of recombinant viruses are selected as candidate virus vaccine strains, on the basis of their high growth yields in eggs and because they have surface antigens relevant to presently circulating viruses, one is left with the problem of determining the degree of virulence of these recombinant clones. This is not a problem when one considers using a high growing recombinant in the production of an inactivated vaccine; however, it is a fundamental problem in selecting a progeny virus as a candidate virus strain to make a live vaccine. The likelihood is that the progeny viruses will be less virulent than the virulent parent after

recombination with avirulent virus; however, the degree of virulence may still be unacceptable for use as a vaccine (1) and it is theoretically possible that recombinant offspring could be more virulent than the parental strains.

The only method presently available to determine the virulence or degree of attenuation for man of the recombinant progeny virus strains is by infecting man. This has been done with several recombinants of recently isolated virulent Influenza A H3N2 viruses and older, less virulent H0N1 or H2N2 viruses. These studies (1, 6) have indicated that attenuation of the virulent virus usually occurs during recombination, but the degree is variable and, at present, unpredictable.

Experiments were performed in mice to determine the degree of virulence of progeny viruses when a strain of virus attenuated for man, but virulent in mice, was recombined with virus strains which were virulent for man but not mice. The strains used were the A/PR/8/34 Mt Sinai (H0N1) virus which grows to high titer in eggs, is virulent in mice but not in man (1), and three recently isolated H3N2 viruses (A/England/42/72, A/Port Chalmers/5/73 and A/Scotland/840/74) and also recombinant progeny of these H0N1 and H3N2 viruses prepared by E.D. Kilbourne or G.C. Schild. Figure 1 demonstrates the recombination procedure and the selection of progeny virus with H3N2 antigens; however, the recombinant viruses possess undetermined virulence for man or mouse. The recombinants supplied by Drs Kilbourne and Schild had been selected by terminal dilution techniques in an effort to provide a virus with a high growth yield. In addition to these recombinants, we tested the 'Alice' strain, a virus prepared from a recombinant of A/England/42/72 (H3N2) and A/PR/8/34 Mt Sinai (H0N1), which then was manipulated to make it resistant to serum inhibitors. This strain has been used in field trials as a candidate live influenza vaccine (7).
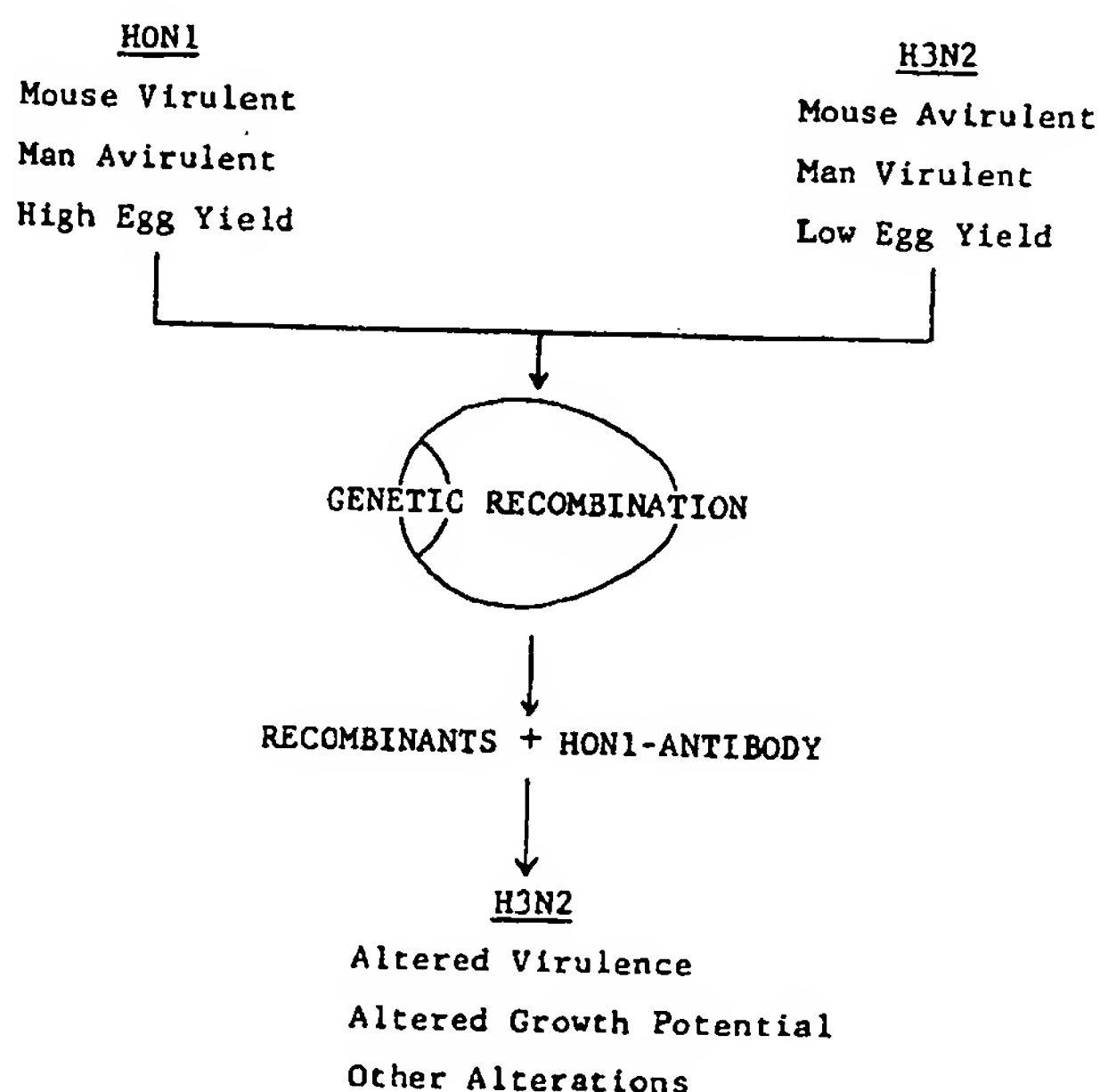

Fig. 1. Alteration of influenza A viruses by genetic recombination.

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Table I summarizes infectivity titers expressed as the 50% egg infectious dose/0.1 ml (EID_{50}) and the 50% mouse lethal dose (LD_{50}) which was determined by inoculating intranasally three-week-old Swiss mice, obtained from the National Institutes of Health, with 0.05 ml of virus diluted ten-fold from 10^1 to 10^5 with phosphate buffered saline pH 7.2, using ten mice per dilution. None of the parent H3N2 viruses killed mice, but the A/PR/8/34 Mt Sinai (HON1) parent virus had an LD_{50} titer of $10^{3.5}$. The EID_{50} of the H3N2 parent viruses was between $10^{6.5}$ and $10^{7.2}$ and the HON1 parent was $10^{8.5}$. The LD_{50} and the EID_{50} of the recombinant offspring varied. Most values were intermediate between the two parents but there were exceptions e.g., X-37 was not lethal in mice (as its H3N2 parent) but X-41 was as lethal in mice as its HON1 parent. There is no relationship between the egg infectivity titers and the virulence of the recombinants in mice. An example is the X-45 recombinant, which lost titer in shipment, and was lethal to mice, although its A/Scotland/840/74 parent was not. Recombinants with approximately the same virus titers varied from 10 to 1000-fold in their ability to kill mice. Mice died with pulmonary consolidation from days 2 to 9 after receiving the various recombinants, similar to mice which were infected with the A/PR/8/34 Mt Sinai parent virus. None of the three recent strains (H3N2) used as parent viruses were lethal for mice. This was expected because influenza viruses, types A and B, must be successively passed in mice lungs in order to produce extensive pulmonary consolidation and death. Thus, in one recombination step, H3N2 viruses became lethal for mice, whereas passage of these viruses without recombination with a virulent strain takes many mouse passages before they become lethal.

Table I. Effect of recombination of A PR/8/34 and influenza A (H3N2) viruses on mouse virulence

Parent	LD_{50}^{*}	EID_{50}^{**}	Recombinant	LD_{50}	EID_{50}
A/PR/8/34 Mt Sinai (HON1)	3.5	8.5	–	–	–
A/England/42/72 (H3N2)	<0.5	7.2	X-37	<0.5	7.2
			"Alice"	2.0	6.5
A/Port Chalmers/1/73 (H3N2)	<0.5	6.5	X-41	3.3	7.5
			MRC-9	3.0	7.5
			MRC-11	2.2	7.5
A/Scotland/840/74 (H3N2)	<0.5	7.2	X-45	2.5	6.8

*Dose of virus (log_{10}) lethal to 50% of 3-week-old mice (NIH Swiss Mice) given 0.05 ml. intranasally.

**Dose of virus (log_{10}) which infects 50% of 10-day-old embryonated hens' eggs.

Another facet of these studies resulted from an attempt to compare the immunogenicity and protection induced by the live 'Alice' vaccine to that induced by a licensed inactivated vaccine. Groups of mice were immunized with 'Alice' vaccine or the inactivated vaccine. At that time, we were aware that the 'Alice' strain had been

passaged in the presence of animal serum to make it serum-inhibitor resistant; however, we were not aware that the history of the 'Alice' strain had included a prior recombination step with A/PR/8/34 Mt Sinai. Following immunization intranasally with 'Alice' vaccine, mice died with extensive pulmonary consolidation. None of the mice died in the control groups, or in the inactivated vaccine group. We then performed an experiment to determine the LD_{50} with 'Alice' vaccine and its parent viruses in mice. As expected, the A/England/42/72 parent did not kill mice and the A/PR/8/34 Mt Sinai parent did, with an LD_{50} of $10^{3.5}$. The recombinant 'Alice' virus had an intermediate LD_{50} of $10^{2.0}$. Undoubtedly, the recombination step which was performed to produce a high yielding strain, resulted in the 'Alice' progeny with newly acquired virulence for mice. This same recombination step could also result in progeny virus that would be less virulent for man than the H3N2 parent because the H0N1 parent is not virulent in man.

The characteristics of the virus which was present in the lungs of 'Alice' infected mice is summarized in Table II. There was 10^5 EID_{50} of virus recovered from the consolidated lungs of the 'Alice' infected mice. This virus was used as antigen in a hemagglutination-inhibition test with antisera specific for its H3N2 and H0N1 parents. Antisera against the H3N2 parent inhibited the virus antigen, but antiserum against the H0N1 parent did not. Serum obtained from mice which survived 'Alice' vaccination possessed high titers of anti-H3N2 antibody, but did not have antibodies against the H0N1 parent. These mice were protected against challenge with a mouse-adapted strain of A/England/42/72. Thus, the recombinant virus which infected these mice, caused pulmonary consolidation and induced antibodies, was antigenically A/England/42/72 (H3N2).

Table II. Characteristics of virulent virus in 'Alice' infected mice

Antigens*	Serum-Inhibitor**	Antibody Induced***
H3N2	Resistant	H3N2

*H3N2 specific antiserum diluted 1:512 inhibited the hemagglutinin of isolate; H0N1 specific antiserum did not inhibit the isolate at a 1:8 dilution.

**Incubated at 36°C for 30 minutes with undiluted heated guinea pig sera, titer was stable (128→256); A/England/42/72 parent was sensitive (32→ <2) compared to antigen incubated with buffered saline.

***Using H3N2 antigen, HI titer was 512; HI titer was <8 with H0N1 antigen.

The other characteristic of the virus isolated from the lungs of the 'Alice' infected mice which we studied was its sensitivity to inhibitors present in guinea pig serum. 'Alice' virus had been prepared by passage of the A/PR/8/34 Mt Sinai and A/England/42/72 recombinant in serum containing inhibitors because it had been reported that inhibitor resistant virus may be less virulent for man. The virus isolated from 'Alice' infected mice was resistant to the serum inhibitors present in guinea pig serum. The hemagglutinin titer of 'Alice's' A/England/42/72 parent fell from 1:32 to

<1:2, but the virus in the lungs of 'Alice' infected mice had a stable titer in the presence of guinea pig serum.

These experiments demonstrate that genetic recombination between influenza A viruses results in recombinant progeny with unpredictable degrees of virulence in an animal model system. Beare and Hall (1971) and McCahon and Schild (1972) have published similar results using recombinants of A/PR/8/34 and A/England/939/69. They determined that progeny virus developing from the recombination of these parents was very variable in virulence for man, and that the recombinant viruses were quite variable in their ability to induce pulmonary consolidation in mice. Moreover, their studies indicated that there was no association between virulence or attenuation in mice and in man.

These results indicate that there are many possible degrees of virulence in progeny virus resulting from recombining avirulent and virulent parental influenza A viruses. There is no available laboratory marker to predict the degree of virulence of recombinant viruses which may be selected for candidates for live virus vaccines. It is clear that recombination between influenza A viruses can dramatically alter the virulence of H3N2 strains for mice and man; therefore, recombinant virus must be characterized before subsequent attempts at attenuation can be evaluated.

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General discussion

Chairman (G.C. Schild, U.K.) : Are there any questions for Dr Ennis ?

W.J. Bogaerts (The Netherlands) : I am a layman in influenza virology, but two questions arise. First, why do you put so much emphasis on the mouse virulence ? I think that the pathogenesis of the infection in man and mouse is different.

Secondly, pathogenesis is in general a factor that is caused by multiple genetic factors, so it is not an easy, stable genetic trait to study.

The next question is, why are you — and not you alone — always using the limiting dilution technique for the isolation of clones ? I thought that we now had better methods of plaque production, and you may be able to work much more easily with a plaque method that gives better isolation and better results on the properties of the variants.

F.A. Ennis : I agree that pathogenesis is complicated and difficult to study. The point of these studies was that if several things are being done to a virus in the process of attempting to attenuate it, I think that a step such as recombination — which clearly can alter the ability of the virus to grow in eggs and alter its pathogenesis for animals, including man — should be evaluated before subsequent steps, such as passage at various temperatures or in the presence of various inhibitors, can be evaluated. As we said, it is necessary at this point because of the lack of laboratory markers (and I did not mean to say that the mouse was a laboratory marker of pathogenicity in man, quite the contrary, the data indicates that the pathogenicity of these recombinants in mice and men is not well correlated) means that no conclusions can be made regarding mechanisms of attenuation if several steps have been performed and the results of one step have not been analysed in the subject in which you wish to use the vaccine, in this case man.

I think that I will refer the last question to Dr Schild who was kind enough to supply us with these recombinants, and to Dr Kilbourne, regarding the selection of recombinants with plaque methods versus egg-yielding virus.

Chairman : I think the answer to that question is that we have no universally applicable and efficient method of plaquing influenza viruses. Some plaque systems are available, but the number of infectious particles required to produce a plaque is of the order of 1000, or even more, so that this method is really not applicable to detailed genetic studies.

F.A. Ennis : This is especially so, I think, with recently isolated virus which you would like to grow to high titre relatively soon for use as a possible candidate vaccine. These viruses generally do not plaque but have to be passed.

W. Hennessen (West Germany) : I am not quite sure whether I understood you correctly. When you showed the results of 'Alice' virus being pathogenic for mice, was this obtained with the 'Alice' virus, or was it obtained with the 'Alice' after recombination with something else ?

F.A. Ennis : This was obtained with 'Alice' vaccine, and 'Alice' vaccine is prepared from A/England virus which had been recombined with APR-8 and then passed in the presence of inhibitors. So it was the vaccine itself that was tested.

Chairman : If I may make a comment on the question of the relationship of mouse and human pathogenicity, I think that was a rather naive hope that there would be some clear-cut relationship. However, as many people suspected at that time, the situation is complicated and, of course, virulence is a multi-gene phenomenon. But I think there is some hope that we may be able to develop techniques for recognizing characteristics related to virulence by biochemical analyses. It has now been clearly shown — and this was described in Madrid recently at the Congress of Virology — that the matrix protein and nucleoprotein of different influenza A viruses have a slightly different biochemical composition, and these two internal components of the virus may be closely related to replication. It may therefore be possible in the future to determine whether these two structural components of a recombinant influenza A virus were derived from one parent or the other, and this may be a great help in establishing the likelihood of virulence characteristics being inherited from one or the other parent.